Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in Lactobacillus plantarum AKU 1009a

AUTHOR(S):

Takeuchi, Michiki; Kishino, Shigenobu; Park, Si-Bum; Kitamura, Nahoko; Ogawa, Jun

CITATION:

Takeuchi, Michiki ...[et al]. Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in Lactobacillus plantarum AKU 1009a. Journal of Molecular Catalysis B: Enzymatic 2015, 117: 7-12

ISSUE DATE:

2015-04-06

URL:

http://hdl.handle.net/2433/198740

Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a

Running title: Hydroxy fatty acid dehydrogenase from *L. plantarum*

Michiki Takeuchi[1], Shigenobu Kishino[1,2,*], Si-Bum Park[2], Nahoko Kitamura[1], Jun Ogawa[1,3,*,†]

[1]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto, 606-8502 Japan,

[2]Laboratory of Industrial Microbiology, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto, 606-8502 Japan

[3]Research Unit for Physiological Chemistry, Kyoto University, Kyoto, Japan

*These corresponding authors contributed equally to this work.

[†]Corresponding author. Tel.: +81 75 753 6115, Fax: +81 75 753 6113.

E-mail address: ogawa@kais.kyoto-u.ac.jp (J. Ogawa).

ABSTRACT

Hydroxy fatty acid dehydrogenase, which is involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a, was cloned, expressed, purified, and characterized. The enzyme preferentially catalyzed NADH-dependent hydrogenation of oxo fatty acids over NAD^+-dependent dehydrogenation of hydroxy fatty acids. In the dehydrogenation reaction, fatty acids with an internal hydroxy group such as 10-hydroxy-*cis*-12-octadecenoic acid, 12-hydroxy-*cis*-9-octadecenoic acid, and 13-hydroxy-*cis*-9-octadecenoic acid served as better substrates than those with α- or β-hydroxy groups such as 3-hydroxyoctadecanoic acid or 2-hydroxyeicosanoic acid. The apparent K_m value for 10-hydroxy-*cis*-12-octadecenoic acid (HYA) was estimated to be 38 μM with a k_{cat} of $7.6 \cdot 10^{-3}$ s^{-1}. The apparent K_m value for 10-oxo-*cis*-12-octadecenoic acid (KetoA) was estimated to be 1.8 μM with a k_{cat} of $5.7 \cdot 10^{-1}$ s^{-1}. In the hydrogenation reaction of KetoA, both (*R*)- and (*S*)- HYA were generated, indicating that the enzyme has low stereoselectivity. This is the first report of a dehydrogenase with a preference for fatty acids with an internal hydroxy group.

Keywords: Lactic acid bacteria; Hydroxy fatty acid; Oxo fatty acid; short-chain dehydrogenase/reductase

1. Introduction

Functional lipids have attracted attention both nutritionally and pharmaceutically. Conjugated linoleic acid (CLA) is a representative functional fatty acid, which has beneficial effects such as decreasing body fat content [1] and preventing tumorigenesis [2,3] and arteriosclerosis [4]. Oxo fatty acids as well as CLA have also been proven to have novel physiological functions. For example, it has recently been reported that 13-oxo-9,11-octadecadienoic acid in tomato juice acts as a potent peroxisome proliferator activated receptor α (PPARα) agonist and improves dyslipidemia and hepatic steatosis induced by obesity [5].

In our previous study, we revealed polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a [6], which is a strain with a potential to produce CLA from linoleic acid [7–10]. The novel saturation metabolism consisted of four enzymes: CLA-HY (hydratase/dehydratase) [6,11,12], CLA-DH (dehydrogenase), CLA-DC (isomerase), and CLA-ER (enone reductase) [6,12]. This saturation metabolism included some oxo fatty acids, such as 10-oxo-*cis*-12-octadecenoic acid (KetoA), 10-oxooctadecanoic acid, and 10-oxo-*trans*-11-octadecenoic acid, as intermediates. These oxo fatty acids are expected to have new physiological activities. CLA-DH generated these oxo fatty acids through dehydrogenation of the corresponding

hydroxy fatty acids, *e.g.*, dehydrogenation of HYA to KetoA.

In this study, we describe the enzymatic and physiochemical characteristics of CLA-DH, which is involved in the saturation metabolism and catalyzes the dehydrogenation of hydroxy fatty acids and the hydrogenation of oxo fatty acids.

2. Materials and methods

2.1. Chemicals

HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid, (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, and 13-hydroxy-*cis*-9-octadecenoic acid were prepared as previously described [6,11,13,14]. Oxo fatty acids (KetoA, 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid, 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid, 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid) were prepared from hydroxy fatty acids (HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid, (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, ricinoleic acid, and 13-hydroxy-*cis*-9-octadecenoic acid) by Jones oxidation, which is oxidation of the

hydroxy group with CrO_3 [15]. (*R*)-HYA was purified from racemic HYA using HPLC equipped with a chiral column in the same method as “Enantiomeric purity analysis of hydroxy fatty acids” shown in below. Fatty acid-free (<0.02%) bovine serum albumin (BSA) was purchased from Sigma (St. Louis, USA). All other chemicals were of analytical grade and were commercially obtained.

2.2. Preparation of CLA-DH

Escherichia coli Rosetta2/pCLA-DH [12] cells were cultured in 1.5 L of Luria-Bertani (LB) medium at 37°C for 2 h with simultaneous shaking at 100 rpm, and then isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After adding IPTG, the transformed cells were cultivated at 20°C for 8 h with simultaneous shaking at 100 rpm. After cultivation, the transformed cells (8 g) were harvested, suspended in a standard buffer (16 mL), and treated with an ultrasonic oscillator (5 min, 4 times, Insinator 201 M; Kubota, Japan). The standard buffer contained 1 mM DTT and 10% (v/v) ethylene glycol in 20 mM potassium phosphate buffer (KPB) (pH 6.5). The cell debris was removed by centrifuging at 1,700*g* for 10 min. The resulting supernatant solutions were used as cell-free extracts. The cell-free extracts were fractioned ultracentrifugation at 100,000*g* for 60 min and the supernatant

was obtained. CLA-DH was purified from this supernatant using a fast protein liquid chromatography (FPLC) system (GE Healthcare) equilibrated with the standard buffer. The supernatant was applied to a HiLoad 26/60 Superdex 200 prep-grade column (GE Healthcare) that had already been equilibrated with standard buffer and eluted. CLA-DH was further purified using a Mono Q 10/100 GL column (GE Healthcare), a Superdex 200 10/300 GL column (GE Healthcare), and a Phenyl Superose HR 10/10 (Pharmacia). The purified CLA-DH was dialyzed with the standard buffer including 50% (v/v) glycerol and stored at −20°C until further use.

2.3. Determination of the molecular mass of CLA-DH

In order to determine the native molecular mass of CLA-DH, the enzyme solution was subjected to high performance gel-permeation chromatography on a G-3000SW column (0.75 × 60 cm, Tosoh, Tokyo, Japan) at room temperature. It was eluted with 100 mM KPB (pH 6.5) containing 100 mM Na_2SO_4 at a flow rate of 0.5 mL/min. The absorbance of the effluent was monitored at 280 nm. The molecular mass of the enzymes was determined from their mobility relative to those of standard proteins.

2.4. Reaction conditions

All operations were performed in an anaerobic chamber. The standard reaction conditions were as described. The reactions were performed in test tubes (16.5 × 125 mm) that contained 1 mL of reaction mixture (20 mM sodium succinate buffer, pH 4.5) with 0.1% (w/v) HYA or KetoA complexed with BSA [0.02% (w/v)] as the substrate, 5 mM NAD^+ or NADH and 42 µg (= 0.04 U/ml) purified CLA-DH. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 µmol of HYA per minute. The reactions were performed under anaerobic conditions in a sealed chamber with an O_2-absorbent (Anaeropack "Kenki," Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan) and gently shaken (120 strokes/min) at 37°C for 15 min. All experiments were performed in triplicate. Reactions were performed under the standard reaction conditions with some modifications, as described below. The optimal reaction temperature was determined by incubating 1 mL of the reaction mixture (20 mM sodium succinate buffer, pH 4.5) at various temperatures for 15 min under anaerobic conditions. The optimal reaction pH was determined at 37°C using 1 mL of 20 mM sodium citrate buffer (pH 3.0–4.0) or 20 mM sodium succinate buffer (pH 4.0–5.5). Thermal stability was determined by measuring the enzyme activity after incubating 1 mL of reaction mixture containing 20 mM sodium succinate buffer (pH 4.5) at various temperatures for 15 min under anaerobic conditions. The pH stability was determined

125 by measuring enzyme activity after incubating at 37°C for 10 min in the following
126 buffers under anaerobic conditions: sodium citrate buffer (50 mM; pH 3.0–4.0), sodium
127 succinate buffer (50 mM; pH 4.0–6.0), KPB (50 mM; pH 5.5–8.0), and Tris-HCl buffer
128 (50 mM; pH 7.0–9.0).
129
130 **2.5. Kinetic analysis**
131 All procedures were performed in an anaerobic chamber. Reactions were
132 performed under standard reaction conditions with modified substrate and enzyme
133 concentrations. The kinetics of HYA dehydrogenation were studied using 30–1000 μM
134 HYA complexed with 0.02% (w/v) BSA as the substrate, 7 μg/mL CLA-DH, and a
135 reaction time of 15 min. The kinetics of KetoA dehydrogenation were studied using
136 1–20 μM KetoA complexed with 0.02% (w/v) BSA as the substrate, 0.35 μg/mL
137 CLA-DH, and a reaction time of 5 min. The kinetic parameters were calculated by using
138 the experimental data with the Michaelis–Menten equation using KaleidaGraph 4.0
139 (Synergy Software Inc., PA, USA).
140
141 **2.6. Lipid analysis**
142 Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an

internal standard. Lipids were extracted from 1 mL of the reaction mixture using 5 mL of chloroform/methanol/1.5% (w/v) KCl in H_2O (2:2:1, by volume) according to the procedure of Bligh-Dyer, and then concentrated by evaporation under reduced pressure [16]. The resulting lipids were dissolved in 5 mL of benzene/methanol (3:2, by volume) and methylated with 300 μL of 1% trimethylsilyldiazomethane (in hexane) at 28°C for 30 min. After methyl esterification, the resulting fatty acid methyl esters were concentrated by evaporation under reduced pressure. The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector, a split injection system, and a capillary column (SPB-1, 30 m × 0.25 mm I.D., SUPELCO, PA, USA). The initial column temperature 180°C (for 30 min) was subsequently increased to 210°C at a rate of 60°C/min, and then maintained at 210°C for 29.5 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty acid peaks were identified by comparing the retention times to those of known standards.

2.7. Enantiomeric purity analysis of hydroxy fatty acids

The enantiomeric purity of HYA, which was produced from KetoA hydrogenation

with CLA-DH, was analyzed using HPLC (Shimadzu, Kyoto, Japan) using a Shimadzu LC 20A System (Shimadzu) equipped with a chiral column (Chiralpak IA, 4.6 mm I.D., Daicel, Osaka, Japan) and an Evaporative Light Scattering Detector System (Shimadzu, Kyoto, Japan) as a detector. Acetonitrile/0.2% formic acid (65:35) was used as a solvent at a flow rate of 1.0 mL/min. Racemic HYA prepared from KetoA reduction with $NaBH_4$ [17] was used as the standard.

3. Results

3.1. Purification of CLA-DH

The recombinant CLA-DH without the tag was purified to homogeneity from cell-free extracts of the transformed *E. coli* through four steps of column chromatography. The purified CLA-DH displayed a single band on an SDS-PAGE gel (Fig. 1). The observed molecular mass of the subunit was 40 kDa, corresponding to a calculated mass of 32 kDa deduced from the amino acid sequence of its gene. The relative native molecular mass was estimated to be 32 kDa by HPLC on a G-3000SW column, indicating that the enzyme consists of the single subunit. The purified CLA-DH was used for further characterization.

3.2. Effects of reaction conditions

CLA-DH required NAD^+/NADH as a cofactor but not $NADP^+$/NADPH. The effects of NAD^+/NADH concentration were examined from 0 to 7.5 mM (Fig. 2a). The dehydrogenation and hydrogenation activities increased with increasing concentrations of NAD^+/NADH. The effects of temperature were also examined. The optimal reaction temperature was found to be 52°C (Fig. 2b). The effects of pH were examined over a pH range from 3.0 to 5.5 with an optimal reaction pH determined to be pH 4.5 (Fig. 2c).

3.3. Enzyme stability

The thermal stability of the purified enzyme was investigated from 18°C to 67°C. The enzyme was incubated at each temperature for 15 min at pH 4.5. More than 80% of the initial activity remained at temperatures up to 28°C (Fig. 3a). The pH stability of the purified enzyme was investigated by incubating the enzyme in different buffers within a pH range of 3.0 to 9.0 for 10 min at 37°C. More than 80% of the initial activity remained in a pH range from 4.5 to 7.5 (Fig. 3b).

3.4. Substrate specificity

In the dehydrogenation reaction, 10-, 12-, or 13-hydroxy C18 fatty acids such as

HYA, (*R*)-HYA, 10-hydroxyoctadecanoid acid, 10-hydroxy-*trans*-11-octadecenoic acid, (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, (*R*)-12-hydroxy-*cis*-9-octadecenoic acid, and 13-hydroxy-*cis*-9-octadecenoic acid served as good substrates and transformed into corresponding 10-, 12-, or 13-oxo fatty acids. In addition, HYA methyl ester and 8-hexadecanol were dehydrogenated to KetoA methyl ester and 8-hexadecanone, respectively. In contrast, 2- or 3-hydroxy fatty acids such as 3-hydroxyoctadecanoic acid, 3-hydroxytetradecanoic acid, and 2-hydroxyeicosanoic acid were not dehydrogenated (Table 1).

In the hydrogenation reaction, 10-, 12- or 13-oxo C18 fatty acids such as KetoA, 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid, 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid, 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid served as good substrates and transformed into corresponding 10-, 12- or 13-hydroxy fatty acids. In addition, KetoA methyl ester and 7-hexadecanone were hydrogenated to HYA methyl ester and 7-hexadecanol, respectively (Table 2).

3.5. Kinetic analysis of the CLA-DH catalyzing reactions

The substrate concentration-reaction velocity curves for HYA dehydrogenation and KetoA hydrogenation were used with the Michaelis–Menten equation. The apparent K_m value for HYA in the dehydrogenation reaction was estimated to be 38 μM with a k_{cat} of $7.6 \cdot 10^{-3}$ sec^{-1}. The apparent K_m value for KetoA in the hydrogenation reaction was estimated to be 1.8 μM with a k_{cat} of $5.7 \cdot 10^{-1}$ sec^{-1}.

3.6. Enantiomeric purities of the hydroxy fatty acids produced by CLA-DH

The enantiomeric purity of HYA produced from KetoA by CLA-DH was analyzed using HPLC with a chiral column. Almost the same amounts of both enantiomers of (*R*)-HYA and (*S*)-HYA were produced from KetoA, indicating that CLA-DH had low stereoselectivity in oxo fatty acid hydrogenation.

3.7. Effects of chemicals on the enzyme activity

The effects of metal ions and inhibitors (1 mM) were investigated in both the hydration and dehydration reactions. The reactions were strongly inhibited by Ag^{+}, Cu^{2+}, Hg^{2+}, VO_3^{-}, WO_4^{2-}, and aluminon (data not shown). 2,3,5-Triphenyltetrazolium inhibited only dehydrogenation activity.

4. Discussion

We revealed polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU 1009a and identified CLA-DH. The CLA-DH gene was located together with CLA-DC (fatty acid isomerase) and CLA-ER (fatty acid enone reductase) genes involved in polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU 1009a [6]. These results suggested that CLA-DH plays an important role in saturation metabolism.

CLA-DH, which belongs to the short-chain dehydrogenase/reductase (SDR) family, showed considerable similarity with other SDRs (Fig. 4). In this paper, we characterized CLA-DH from the aspect of its physiological function to clarify its distinct characteristic properties in the SDR family, especially from the viewpoint of substrate specificity. There are few reports regarding either hydroxy fatty acid dehydrogenation or oxo fatty acid hydrogenation in the SDR family. However, CLA-DH catalyzed the dehydrogenation or hydrogenation of fatty acids which have an internal hydroxy or an oxo group, respectively (Table 1 and 2). *Micrococcus luteus* WIUJH-20 was reported to convert 10- or 12-hydroxyoctadecanoic acid to the corresponding oxooctadecanoic acid. The amino acid sequence of the enzyme which catalyzes the above oxidation of hydroxy fatty acid in *M. luteus* WIUJH-20 belongs to a secondary alcohol dehydrogenase [18]. The amino acid sequence of the secondary alcohol dehydrogenase

from *M. luteus* did not resemble that of CLA-DH, indicating that the dehydrogenation activity of CLA-DH was characteristic activity among the SDR family.

As a characteristic property of CLA-DH, the enzyme showed higher activity in hydrogenation than dehydrogenation reactions. The activity of KetoA hydrogenation was 5 times higher than that of HYA dehydrogenation (Fig. 2).

Although many SDRs have high enantioselectivity [19-23], CLA-DH had low enantioselectivity to dehydrogenate both (*R*) and (*S*) hydroxy fatty acids (Table1) and produce (*R*) and (*S*) hydroxy fatty acids from oxo fatty acid. In addition, CLA-DH dehydrogenated 10-, 12-, and 13-hydroxy fatty acids (Table 1), indicating its low regioselectivity.

In our previous study, we reported the production of many kinds of hydroxy fatty acids such as 10- and 13-hydroxy octadecapolyenoic acid [6,11,13,14]. Using these various hydroxy fatty acids and hydroxy fatty acid dehydrogenase such as CLA-DH, we can provide many kinds of corresponding oxo fatty acids by applying the wide substrate specificity of CLA-DH. These results enable us to provide new functional lipids, oxo fatty acids.

5. Conclusions

269 The properties of CLA-HY, a novel hydroxy fatty acid dehydrogenase from *L.*
270 *plantarum* were investigated. CLA-DH showed wide substrate specificity toward
271 hydroxy fatty acids with a preference to those with an internal hydroxy group. Such
272 substrate preference explained well that CLA-DH is involved in polyunsaturated fatty
273 acid saturation metabolism. From an application oriented perspective, CLA-DH is
274 useful for the production of oxo fatty acids with unique physiological functions in
275 combination with fatty acid hydratases such as CLA-HY (11, 13), which were reported
276 as good catalysts generating hydroxy fatty acids from common C18 fatty acids.
277

278 **ACKNOWLEDGMENTS**

279 This work was supported, in part, by the Industrial Technology Research Grant Program
280 in 2007 (Grant 07A08005a to S.K.); the Project for the Development of a Technological
281 Infrastructure for Industrial Bioprocesses on Research and Development of New
282 Industrial Science and Technology Frontiers (S.S.) from the New Energy and Industrial
283 Technology Development Organization (NEDO) of Japan; Scientific Research Grants in
284 Aid 19780056 (to S.K.), 16688004 (to J.O.), and 18208009 (to S.S.); the Centers of
285 Excellence for Microbial-Process Development Pioneering Future Production Systems
286 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the

Bio-Oriented Technology Research Advancement Institution of Japan (J.O.); Science and Technology Promotion Program for Agriculture Forestry, Fisheries and Food Industry from the Ministry of Agriculture, Forestry and Fisheries of Japan (J.O.); Advanced Low Carbon Technology Research and Development Program of Japan (S.K.); M.T. and S.K. received Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

[1] Y. Park, K.J. Albright, W. Liu, J.M. Storkson, M.E. Cook, M.W. Pariza, Lipids. 32 (1997) 853–858.

[2] M.W. Pariza, Y.L. Ha, in: Y. Kuroda, D. Shankel, M.D. Waters (Eds.), Antimutagenesis and Anticarcinogenesis Mechanism II, Plenum, New York, 1990, pp. 167–170.

[3] C. Ip, S.F. Chin, J.A. Scimeca, M.W. Pariza, Cancer Res. 51 (1991) 6118–6124.

[4] K.N. Lee, D. Kritchevsky, M.W. Pariza, Atherosclerosis. 108 (1994) 19–25.

[5] Y.I. Kim, S. Hirai, T. Goto, C. Ohyane, H. Takahashi, T. Tsugane, C. Konishi, T. Fujii, S. Inai, Y. Iijima, K. Aoki, D. Shibata, N. Takahashi, T. Kawada, PLoS One. 7 (2012) e31317.

305 [6] S. Kishino, M. Takeuchi, S.B. Park, A. Hirata, N. Kitamura, J. Kunisawa, H. Kiyono,
306 R. Iwamoto, Y. Isobe, M. Arita, H. Arai, K. Ueda, J. Shima, S. Takahashi, K. Yokozeki,
307 S. Shimizu, J. Ogawa, Proc. Natl. Acad. Sci. USA. 110 (2013) 17808–17813.
308 [7] J. Ogawa, S. Kishino, A. Ando, S. Sugimoto, K. Mihara, S. Shimizu, J. Biosci.
309 Bioeng.100 (2005) 355–364.
310 [8] S. Kishino, J. Ogawa, Y. Omura, K. Matsumura, S. Shimizu, J. Am. Oil Chem. Soc.
311 79 (2002) 159–163.
312 [9] S. Kishino, J. Ogawa, A. Ando, T. Iwashita, T. Fujita, H. Kawashima, S. Shimizu,
313 Biosci. Biotechnol. Biochem. 67 (2003) 179–182.
314 [10] S. Kishino, J. Ogawa, K. Yokozeki, S. Shimizu, Biosci. Biotechnol. Biochem. 75
315 (2011) 318–322.
316 [11] M. Takeuchi, S. Kishino, A. Hirata, S.B. Park, N. Kitamura, J. Ogawa, J. Biosci.
317 Bioeng. doi:10.1016/j.jbiosc.2014.10.022
318 [12] S. Kishino, S.B. Park, M. Takeuchi, K. Yokozeki, S. Shimizu, J. Ogawa, Biochem.
319 Biophys. Res. Commun. 416 (2011) 188–193.
320 [13] M. Takeuchi, S. Kishino, K. Tanabe, A. Hirata, S.B. Park, S. Shimizu, J. Ogawa,
321 Eur. J. Lipid Sci. Technol. 115 (2013) 386–393.
322 [14] J. Ogawa, K. Matsumura, S. Kishino, Y. Omura, S. Shimizu, Appl. Environ.

323 Microbiol. 67 (2001) 1246–1252.

324 [15] R.G. Curtis, S.I. Heilbron, E.R.H. Jones, G.F. Woods, J. Chem. Soc. (1953)

325 457–464.

326 [16] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911–917.

327 [17] A.L. Gemal, J.L. Luche, J. Am. Chem. Soc. 103 (1981) 5454–5459.

328 [18] J.W. Song, E.Y. Jeon, D.H. Song, H.Y. Jang, U.T. Bornscheuer, D.K. Oh, J.B. Park,

329 Angew. Chem. 125 (2013) 2594–2597.

330 [19] N. Urano, S. Fukui, S. Kumashiro, T. Ishige, S. Kita, K. Sakamoto, M. Kataoka, S.

331 Shimizu, J. Biosci. Bioeng. 111 (2011) 266–271.

332 [20] N.H. Schlieben, K. Niefind, J. Muller, B. Riebel, W. Hummel, D. Schomburg, J.

333 Mol. Biol. 349 (2005) 801–813.

334 [21] A. Pennacchio, B. Pucci, F. Secundo, F.L. Cara, M. Rossi, C.A. Raia, Appl.

335 Environ. Microbiol. 74 (2008) 3949–3958.

336 [22] N. Itoh, K. Isotani, M. Nakamura, K. Inoue, Y. Isogai, Y. Makino, Appl. Microbiol.

337 Biotechnol. 93 (2012) 1075–1085.

338 [23] Y. Kallberg, U. Oppermann, H. Jornvall, B. Persson, Eur. J. Biochem. 269 (2002)

339 4409–4417.

340

341 **Figure legends**

342 **Fig. 1 SDS-PAGE analysis of purified CLA-DH**

343 Molecular mass standards: from the top, phosphorylase b (97,200), bovine serum
344 albumin (66,400), ovalbumin (45,000), carbonic anhydrase (29,000), and trypsin
345 inhibitor (20,100). The observed molecular weight of purified CLA-DH was 40 kDa.

346 **Fig. 2 Effects of NAD^+/NADH concentrations, temperature, and pH on the activity**
347 **of CLA-DH**

348 (a) Effects of NAD^+/NADH concentrations: Dehydrogenation activity (closed circles)
349 and hydrogenation activity (open circles) were assayed under standard reaction
350 conditions, except for NAD^+/NADH concentrations. (b) Effects of temperature:
351 Dehydrogenation activity (closed circles) and hydrogenation activity (open circles) were
352 assayed under standard reaction conditions, except for the temperature. (c) Effects of
353 pH: Activity was assayed under standard reaction conditions, except for the buffers used.
354 Sodium citrate buffer (closed and open circles for dehydrogenation and hydrogenation,
355 respectively), pH 3.0–4.0, and sodium succinate buffer (closed and open triangles for
356 dehydrogenation and hydrogenation, respectively), pH 4.0–5.5, were used.

357 **Fig. 3 Effects of temperature and pH on stability of CLA-DH**

358 (a) Effect of temperature: The thermal stability of the dehydrogenation activity (closed

359 circles) and hydrogenation activity (open circles) were assessed under standard reaction
360 conditions after incubation at each temperature (18°C –67°C) for 30 min. The activities
361 after incubation at 18°C were defined as 100% for dehydrogenation (0.048 U/mg) and
362 hydrogenation (0.22 U/mg). (b) Effect of pH: The pH stabilities of the dehydrogenation
363 (closed) and hydrogenation (open) reactions were evaluated under standard reaction
364 conditions after incubation at 37°C for 10 min at each pH. Sodium citrate buffer, pH
365 3.0–4.0 (circles), sodium succinate buffer, pH 4.0–6.0 (triangles), potassium phosphate
366 buffer, pH 5.0–7.5 (diamonds), and Tris-HCl buffer, 7.0–9.0 (squares) were used. The
367 activities after incubation in sodium succinate buffer (pH 4.5) were defined as 100% for
368 dehydrogenation (0.042 U/mg) and hydrogenation (0.22 U/mg).

369 **Fig. 4 Multiple-sequence alignment of CLA-DH and ADHs belonging to the SDR**
370 **family**

371 The SDR family includes *Rhodococcus erythropolis* (AADH), *Lactobacillus brevis*
372 (LbRADH), *Thermus thermophilus* (TtADH), and *Leifsonia* sp. strain S749 (LSADH).
373 The accession numbers of the listed proteins are as follows: CLA-DH, BAL42247;
374 AADH, BAF43657; LbRADH, YP_794544; TtADH, YP_003977; LSADH, BAD99642.
375 Black and gray shading indicate residues highly conserved in the SDR family.

376 TGXXXGXG is co-enzyme binding region in typical SDRs. The star indicates the four

377 members of catalytic tetrad.

378

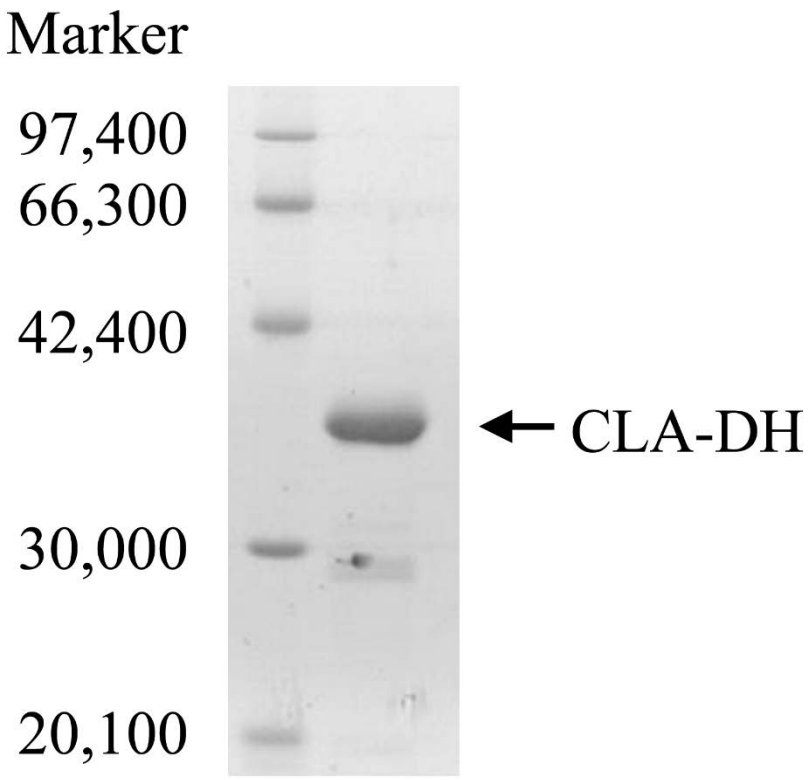

Fig. 1 SDS-PAGE analysis of purified CLA-DH

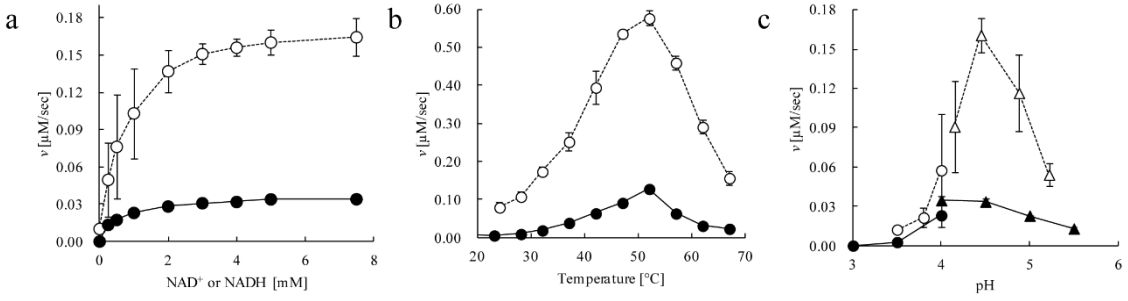

Fig. 2 Effects of NAD$^+$/NADH concentrations, temperature, and pH on the activity of CLA-DH

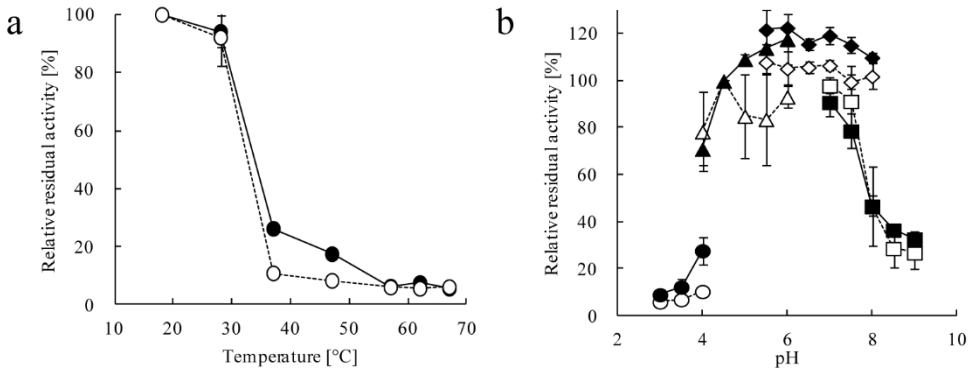

Fig. 3 Effects of temperature and pH on stability of CLA-DH

```
CLA-DH    1 --MKDFKDKVMFITGAAHGFGQVIAEGAADRGMKLTIVDIDEPALKKTYQHILDKGAEVL 58
AADH      1 -MFNSIEGRSVVVTGGSKGIGLGMVRVFARAGANVLMTARDALTLERAAEGLNGLPGAVS 59
LbRADH    1 -MSNRLDGKVAIVTGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEKAAKSVGTPDQIQF 59
TtADH     1 MGLF--AGKGVLVTGGARGIGRAIAQAFAREGALVALCDLRPEGKE--VAEAIGGAFFQV 56
LSADH     1 MAQYDVADRSAIVTGGGSGIGRAVALTLAASGAAVLVTDLNEEHAQAVVAEIEAAGGKAA 60
                            TGXXXGXG

CLA-DH   59 MVTADVTKEASVDDAVEQAMEKFGRIDLLINNAGIALP-GRIWELPTRDWEWIMHINLMS 117
AADH     60 TLQVDVTNPDSLAGMAEVAAERHGGIDVLCANAGIFPS-KRLGEMTSEDMDSVFGVNVKG 118
LbRADH   60 FQHDSSDED-GWTKLFDATEKAFGPVSTLVNNAGIAVN-KSVEETTTAEWRKLLAVNLDG 117
TtADH    57 DLEDERERV-RFVEE---AAYALGRVDVLVNNAAIAAPGSAL-TVRLPEWRRVLEVNLTA 111
LSADH    61 ALAGDVTDP-AFGEASVAGANALAPLKIAVNNAGIGGEAATVGDYSLDSWRTVIEVNLNA 119
                                      *

CLA-DH  118 QVYAMKRVIPIMIQQKTHADILNVASIAGLVDT-PGMPSYHASKFASVGMTEATAYDLQR 176
AADH    119 TIHAVQACMPWLETSGRGRVVVTS-SITGPVTGYPGWSHYGASKAAQMGFIRTAAIELAP 177
LbRADH  118 VFFGTRLGIQRMKNKGLGASIINMSSIEGFV-GDPSLGAYNASKGAVRIMSKSAALDCAL 176
TtADH   112 PMHLSALAAREMRKVG-GGAIVNVASVQGLF-AEQENAAYNASKGGLVNLTRSLALDLAP 169
LSADH   120 VFYGMQPQLKAMAANG-GGAIVNMASILGSV-GFANSSAYVTAKHALLGLTQNAALEYAA 177
                            *                 *   *

CLA-DH  177 ANIDIDMHVMCPGFVQTDLYHTENHRPAQYSDPTDPYYQSEAYLKGQQFAKYVITNGKPI 236
AADH    178 KR--ITINAVLPGNVITEG---LDGLGQEY---LDQMASSVPAG----------SLGSVE 219
LbRADH  177 KDYDVRVNTVHPGYIKTP--LVDDLPGAE---EAMSQRTKTPMG----------HIGEPN 221
TtADH   170 LR--IRVNAVAPGAIATEAVLEAIALSPDPERTRRDWEDLHALR----------RLGKPE 217
LSADH   178 DK--VRVVAVGPGFIRTP--LVEANLSAD---ALAFLEGKHALG----------RLGEPE 220

CLA-DH  237 DTIADTVFKALEDNRFYILTHPEYNPLIEDRVKRIVTDGAPDVHIMDGIM---        286
AADH    220 DIANAALFFALDEAAYITG-------------QSLIVDGGQVLPESAMALGEL        259
LbRADH  222 DIAYICVYLASNESKFATG-------------SEFVVDGGYTAQ---------        252
TtADH   218 EVAEAVLFLASEKASFITG-------------AILPVDGGMTASFMMAGRPV-        256
LSADH   221 EVASLVAFLASDAASFITG-------------SYHLVDGGYTAQ---------        251
```

Fig. 4 Multiple-sequence alignment of CLA-DH and ADHs belonging to the SDR family

Table 1 **Substrate specificity of CLA-DH for dehydrogenation.**

Substrate	Relative activity [%]
(*S*)-10-Hydroxy-*cis*-12-octadecenoic acid (HYA)	100 [a]
(*R*)-10-Hydroxy-*cis*-12-octadecenoic acid ((*R*)-HYA)	98
10-Hydroxyoctadecanoic acid	25
10-Hydroxy-*trans*-11-octadecenoic acid	69
(*S*)-10-Hydroxy-*cis*-12,*cis*-15-octadecadienoic acid	75
(*S*)-10-Hydroxy-*cis*-6,*cis*-12-octadecadienoic acid	54
(*R*)-12-Hydroxy-*cis*-9-octadecenoic acid	62
13-Hydroxy-*cis*-9-octadecenoic acid	142
3-Hydroxyoctadecanoic acid (C18)	- [b]
3-Hydroxytetradecanoic acid (C14)	-
2-Hydroxyeicosanoic acid (C20)	-
Methyl (*S*)-10-Hydroxy-*cis*-12-octadecenoate	127
8-Hexadecanol	24

[a], The activity of (*S*)-10-hydroxy-*cis*-12-octadecenoic acid dehydrogenation (=0.048 U/mg) under the condition (5 mM NAD^+; 37°C, pH 4.5, 15 min) was defined as 100%.

[b]-, not detected.

Table 2 **Substrate specificity of CLA-DH for hydrogenation.**

Substrate	Relative activity [%]
10-Oxo-*cis*-12-octadecenoic acid (KetoA)	100 [a]
10-Oxooctadecanoic acid	66
10-Oxo-*trans*-11-octadecenoic acid	53
10-Oxo-*cis*-12,*cis*-15-octadecadienoic acid	44
10-Oxo-*cis*-6,*cis*-12-octadecadienoic acid	6
12-Oxo-*cis*-9-octadecenoic acid	87
13-Oxo-*cis*-9-octadecenoic acid	156
Methyl 10-oxo-*cis*-12-octadecenoate	170
7-Hexadecanone	332

[a], The activity of 10-oxo-*cis*-12-octadecenoic acid hydrogenation (=0.22 U/mg) under the condition (5 mM NADH; 37°C, pH 4.5, 15 min) was defined as 100%.